オキサリプラチンによる急性末梢神経障害におけるTRPA1チャネルの関与(Digest_要約)

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２０１３

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謹呈

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拙書ではございますが、御一読頂ければ幸いに存じます。
今後とも御指導、御鞭撻を賜りますようお願い申し上げます。

平成２６年3月２４日

趙　萌

オキサリプラチンによる急性末梢神経障害における TRPA1 チャネルの関与

(Involvement of TRPA1 channel in oxaliplatin-induced acute peripheral neuropathy)

２０１３

趙　萌

Contents

Preface

Peripheral neuropathy is a common side effect of platinum-based chemotherapeutic compounds such as oxaliplatin and cisplatin, taxanes such as paclitaxel and vinca alkaloids such as vincristine. Among them, oxaliplatin, a third-generation, platinum-based chemotherapeutic agent, has superior activity as a first-line treatment in advanced colorectal cancer and as adjuvant treatment. Oxaliplatin has a better safety profile, characterized by lower hematotoxicity and manageable gastrointestinal toxicity, than other platinum-based chemotherapeutics. However, oxaliplatin induces moderate to severe peripheral neuropathy, characterized by acute and chronic, two types of neurological symptoms. The acute neuropathy is specific to oxaliplatin and is exacerbated by cold. While the oxaliplatin-induced chronic peripheral neuropathy can be explained, at least in part, by the accumulation of platinum adducts in the dorsal root ganglia (DRG), the mechanisms underlying the acute peripheral neuropathy are poorly understood.

On the other hand, transient receptor potential (TRP) channels are a family of nonselective cation channels. Some TRP channels are expressed in sensory neurons, including TRPV1, TRPA1, and TRPM8. These receptors are thermosensitive and play a critical role in pain generation. The TRP ankyrin 1 (TRPA1), a calcium-permeable cation channel co-expressed with TRPV1 in a subpopulation of nociceptive primary sensory neurons is activated by pungent ingredients present in an array of spices, including allyl isothiocyanate (mustard, wasabi), cinnamaldehyde (cinnamon), and others. It has been reported that TRPA1 is activated by noxious cold temperature and robust evidence has been accumulated indicating that reactive oxygen species (ROS) and various endogenous byproducts deriving from ROS induced peroxidation of plasma membrane phospholipids gate TRPA1, thereby causing pain and neurogenic inflammation. TRPA1 is a sensor of both cold temperature and oxidative stress. Therefore, I have hypothesized that TRPA1 mediate cold hypersensitivity provoked by oxaliplatin. The present data show that:

In chapter 1, I established a new oxaliplatin-induced acute cold hypersensitivity mouse model. A single intraperitoneal administration of oxaliplatin induced a characteristic acute cold hypersensitivity, while mechanical hypersensitivity was not observed, which is similar with the clinical observation. Then the effects of standard

analgesics on the oxaliplatin-induced cold hypersensitivity were evaluated in this mouse model. I found that gabapentin, mexiletine, tramadol and calcium gluconate significantly inhibited, and morphine and milnacipran decreased the acute cold hypersensitivity, while diclofenac and amitriptyline had no effects. These results suggest that gabapentin, mexiletine and calcium gluconate are effective against oxaliplatin-induced acute peripheral neuropathy.

In chapter 2, the involvements of thermosensitive TRP channels were evaluated. Pre-treatment of oxalipaltin enhanced TRPA1 channel agonist-evoked nocifensive behaviors and $[Ca^{2+}]_i$ responses in cultured DRG neurons, respectively. Moreover, the oxaliplatin-induced cold hypersensitivity was inhibited by a TRPA1 antagonist, HC030031 and TRPA1 knockout mice. The present data suggest that the acute cold hypersensitivity characteristically induced by oxaliplatin could be linked to an enhanced responsiveness of TRPA1, but not TRPM8 and TRPV1, on DRG neurons.

In chapter 3, the mechanisms of TRPA1 activation/sensitization were further investigated. High concentration of oxaliplatin elicited TRPA1 activation directly, while low concentration of oxaliplatin pretreatment enhanced hydrogen peroxide (H_2O_2)-evoked TRPA1 responses. These results suggest oxaliplatin could sensitize TRPA1 function, and subsequently the sensitized TRPA1 is activated by ROS probably produced from oxaliplatin-mediated mitochondrial dysfunction, which may contribute to the oxaliplatin-induced acute peripheral neuropathy.

The detailed results of my work will be described as follows:

Abbreviations

2-APB	2-Aminoethoxydiphenyl borate
AITC	allyl isothiocyanate
ANOVA	analysis of variance
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglia
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol bis(2-aminoethylether)tetraacetic acid
FBS	fetal bovine serum
FOLFOX	Folinic acid + Fluorouracil + Oxaliplatin
Fura 2-AM	Fura 2-acetoxymethylester
GSH	glutathione
H_2O_2	hydrogen peroxide
HEK293	human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
NSAID	non-steroidal antiinflammatory drug
PBN	*N*-*tert*-Butyl-α-phenylnitrone
PBS	Phosphate buffered solution
PG-1	Peroxy Green 1
PHD	Prolyl hydroxylases
ROS	reactive oxygen species
SNRI	selective serotonin noradrenaline reuptake inhibitor
TRP	transient receptor potential
TRPA1	transient receptor potential ankyrin 1
TRPM8	transient receptor potential melastatin 8
TRPV1	transient receptor potential vanilloid 1
cDNA	complementary deoxyribonucleic acid
i.p.	intraperitoneal
i.pl.	intraplantar
i.v.	intravenous
mRNA	messenger ribonucleic acid
s.c.	subcutaneous

Chapter 1 Establishment of oxaliplatin-induced acute peripheral neuropathy in mice

Oxaliplatin, a third-generation, platinum-based chemotherapeutic agent, is a component of the FOLFOX regimen (oxaliplatin + 5-fluorouracil + leucovorin) as a standard adjuvant treatment for advanced colorectal cancer [1, 2]. Oxaliplatin has a better safety profile, characterized by lower hematotoxicity and manageable gastrointestinal toxicity, than other platinum-based chemotherapeutics. However, peripheral neuropathy is a common side effect of platinum-based chemotherapeutic compounds such as oxaliplatin and cisplatin, taxanes such as paclitaxel and vinca alkaloids such as vincristine [3]. Oxaliplatin induces moderate to severe peripheral neuropathy, characterized by two types of neurological symptoms [4, 5]. During or within hours after its infusion, an acute neuropathy, including acral numbness, paresthesia, dysesthesia and pain, develops in almost all patients that intensify over time, causing serious discomfort. After multiple chemotherapy cycles, chronic cumulative peripheral neuropathy, such as sensory loss and motor dysfunction, occurs in 10-15% of treated patients, a rate similar to that of cisplatin [5, 6]. The acute neuropathy is specific to oxaliplatin [4, 5, 7], while effective pharmacological strategy for the management of oxaliplatin-induced acute peripheral neuropathy remains controversial [8-10].

Many studies in animal models focus on the oxaliplatin-induced chronic and/or subacute painful peripheral neuropathy that appear several days to several weeks after oxaliplatin administration [11-13]. However, oxaliplatin-induced acute peripheral neuropathy is poorly characterized in animal models. In chapter 1, I tried to produce a new mouse model which could represent the acute neuropathy characteristic to oxaliplatin and assessed the effects of standard analgesics, such as the non-steroidal anti-inflammatory agent (NSAID), opioid analgesics, tricyclic antidepressant, serotonin and noradrenaline reuptake inhibitor (SNRI), calcium channel α_2-δ ligand, local anesthetic and calcium gluconate [8-10], on the oxaliplatin-induced acute cold hypersensitivity in mice.

Methods

Animals

The male C57BL/6 J mice aged between 6-8 week-old were purchased from Japan SLC (Shizuoka, Japan). All mice were housed under constant ambient temperature (24 ± 1°C) and humidity (55 ± 10%), with alternate light-dark cycles from 8:00 a.m. to 20:00 p.m.. Food and water were freely available. All experiments were conducted in accordance with the Ethical Guidelines of the Kyoto University Animal Experimentation Committee.

Drugs

Oxaliplatin and sodium oxalate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and freshly dissolved in 5% glucose solution. *cis*-Diammineplatinum(II) dichloride (cisplatin) and paclitaxel were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Cisplatin was freshly dissolved in sterile saline, and paclitaxel in Cremophor® EL (Sigma-Aldrich) and dehydrated ethanol (1:1) to obtain a stock solution. Prior to its administration, paclitaxel was further diluted with sterile saline. Mice received a single intraperitoneal (i.p.) administration of oxaliplatin (1, 5 or 10 mg/kg), cisplatin (5 mg/kg), paclitaxel (6 mg/kg), or vehicle. The doses of these chemotherapeutic agents were chosen based on previous reports with a single intraperitoneal administration [11, 13-17].

Morphine hydrochloride (Takeda Pharmaceutical Co., Osaka), tramadol hydrochloride (gift from Nippon Shinyaku, Kyoto), amitriptyline hydrochloride (LKT Laboratories, MN, USA), milnacipran hydrochloride (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diclofenac sodium salt, gabapentin and mexiletine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA) were freshly dissolved in sterile saline. Morphine and tramadol were administrated subcutaneously (s.c.), and milnacipran and mexiletine were administrated intraperitoneally 30 minutes before, and diclofenac, gabapentin and amitriptyline were administered intraperitoneally 1 hour before the behavioral test at a volume of 10 ml/kg. Calcium gluconate monohydrate (Wako) was freshly dissolved in 5% glucose solution, and was infused intravenously (i.v.) just before oxaliplatin administration [7].

Behavioral tests

von Frey filament test

Mechanical sensitivity was assessed by the up-down method using calibrated von Frey filaments, as previously described with slight modifications [18, 19]. Mice were acclimated on a wire-mesh floor in Plexiglas cubicles (9 cm L × 5 cm W × 5 cm H) for 1 h, after which mechanical sensitivity was evaluated using a set of four calibrated von Frey fibers (0.07, 0.16, 0.4, 1.0 g; Stoelting Co., Wood Dale, IL USA) applied to the plantar surface of the left hindpaw for a few seconds until they bent slightly. A withdrawal reflex of the hindpaw during stimulation or immediately after stimulus removal was considered a positive response. If a positive response was obtained to the first stimulus, the 0.16-g filament, then the next lower filament was applied; if there was no response, the next higher filament was used. After the first change in responses, the experiment was continued until four additional responses were obtained. The 50% paw withdrawal threshold value was then calculated [19].

Cold-plate test

Cold sensitivity was assessed with the Hot/Cold Plate (Ugo Basile, Milan, Italy). Mice were allowed to acclimate to the testing apparatus for 1 h, after which they were individually placed on the center of a cold plate maintained at 5°C in a transparent Plexiglas cylinder. Escape behaviors were observed for 60 s and graded with a score of 0 = no response; 1 = moderate effort to avoid cold, such as lifting a hindpaw or walking backwards; and 2 = vigorous effort to escape cold, such as jumping. The sum of the scores recorded within a 60-s period was calculated.

Statistical analysis

The data were analyzed using Graphpad Prism and are presented as means ± S.E.M. Statistical significance was calculated by one-way or two-way analyses of variance (ANOVA), followed by the Bonferroni or Tukey-Kramer post-hoc test. Time-course data were analyzed by two-way ANOVA for repeated measures, followed by the Bonferroni post-hoc test. In all cases, differences of $p < 0.05$ were considered statistically significant.

Results

Effect of oxaliplatin, cisplatin, and paclitaxel on acute mechanical and cold sensitivities

The effects of a single administration of oxaliplatin on behavioral sensitivity to mechanical and cold stimuli were assessed in a von Frey filament test and a cold plate test, respectively (Fig. 1-1). A single intraperitoneal administration of oxaliplatin (5 mg/kg) significantly decreased the 50% withdrawal threshold to mechanical stimulation with von Frey filaments ($F_{1, 14}$ = 16.85, p < 0.01). While a decrease in the mechanical threshold was not apparent 2 h after oxaliplatin administration, the response became significant at 1 day and lasted for at least 7 days, compared with the vehicle-administered group (Fig. 1-1A). In the cold plate test, oxaliplatin (5 mg/kg) significantly increased the escape behavior scores measured in response to cold stimulation ($F_{1,10}$ = 10.06, p < 0.01). Significant increases in the cold escape behaviors were observed after 2 h and lasted for at least 7 days after oxaliplatin administration, compared with the vehicle-administered group (Fig. 1-1B). I assessed the dose-dependent effect of oxaliplatin on the acute cold hypersensitivity in the cold plate test. The cold escape behavior scores were significantly increased 2 h after the administration of oxaliplatin (1, 5 and 10 mg/kg) in a dose-dependent manner ($F_{3,20}$ = 18.57, p < 0.001), and the significant effects were observed at doses of 5 and 10 mg/kg, but not 1 mg/kg, compared with the vehicle-administered group (Fig. 1-1C). Oxaliplatin is metabolized to oxalate and dichloro(1,2-diaminocyclohexane)platinum. As oxaliplatin-induced cold hypersensitivity measured in the acetone test is caused, at least in part, by oxalate [13], I examined the effect of oxalate on the cold sensitivity in the cold plate test. The dose of sodium oxalate (1.7 mg/kg) was calculated from the molecular weight of oxaliplatin included in the oxaliplatin preparation (5 mg/kg). A single intraperitoneal administration of oxalate significantly increased the cold escape behavior scores ($F_{1,12}$ = 14.81, p < 0.01). Significant increases were observed after 2 h and lasted for 3 days after oxaliplatin administration, compared with the vehicle-administered group (Fig. 1-1D).

The repeated administration of cisplatin or paclitaxel is reported to produce mechanical and thermal hypersensitivity, with maximal effects observed several days to several weeks after drug administration [15, 20-22]. In this study, I asked whether

cisplatin and paclitaxel induce an early-phase acute mechanical or cold hypersensitivity. However, 2 h after a single intraperitoneal administration of cisplatin (5 mg/kg) or paclitaxel (6 mg/kg) there was no change in the 50% withdrawal threshold to mechanical stimulation) or in the escape behavior scores measured in response to cold stimulation (Fig. 1-2).

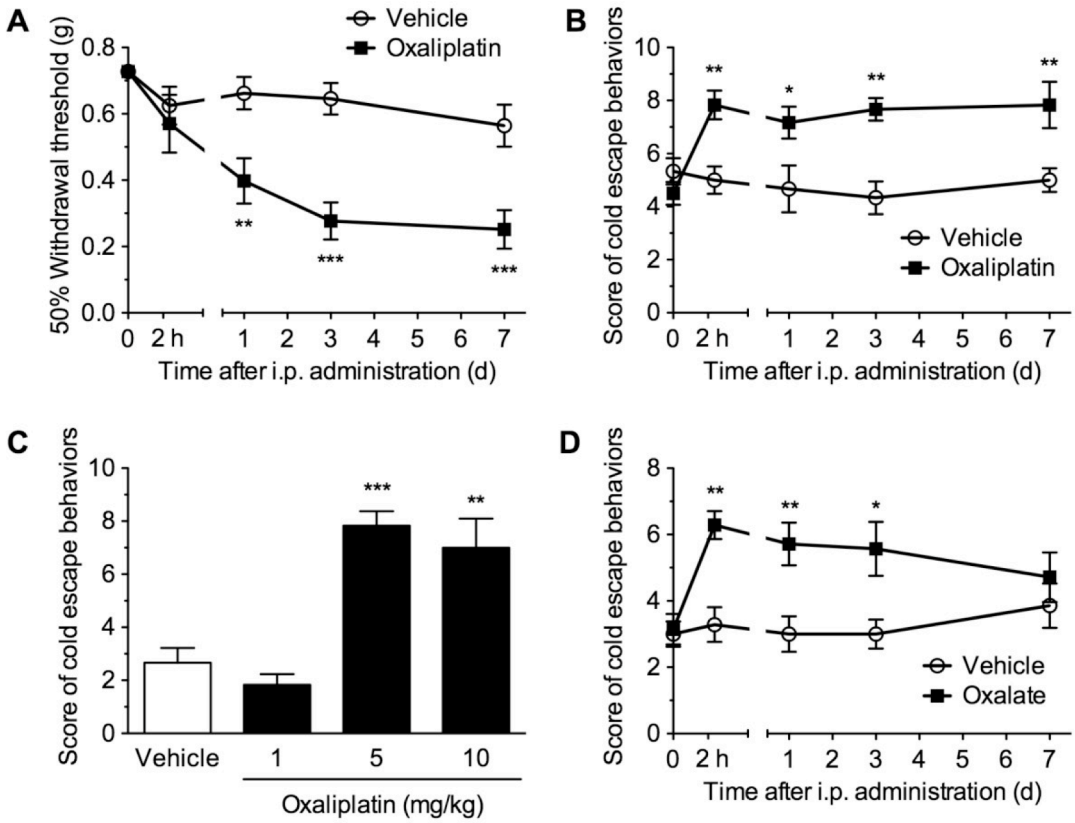

Figure 1-1. A single administration of oxaliplatin induces acute cold hypersensitivity, but not acute mechanical hypersensitivity. (**A**, **B**) Mice were intraperitoneally administered either vehicle or oxaliplatin (5 mg/kg). At the indicated times, the 50% withdrawal threshold to mechanical stimulation and escape behaviors in response to cold stimulation (5°C) were evaluated in a von Frey filament test (**A**, $n = 8$) and a cold plate test (**B**, $n = 6$), respectively. Cold escape behaviors were scored depending on a behavioral assessment, with the total score calculated for a period of 60 s. * $p < 0.05$, ** $p < 0.01$ compared with vehicle-administered group. (**C**) Mice were intraperitoneally administered vehicle or oxaliplatin (1, 5 or 10 mg/kg). Two hours after the administration, the cold escape behaviors were scored in the cold plate test. $n = 6$. ** $p < 0.01$, *** $p < 0.001$ compared with vehicle-administered group. (**D**) Mice were intraperitoneally administered either vehicle or oxalate (1.7 mg/kg). At the indicated times, the cold escape behaviors were scored in the cold plate test. $n = 7$. * $p < 0.05$, ** $p < 0.01$ compared with vehicle-administered group. Data are presented as the means ± S.E.M. Statistical significance was calculated by one-way ANOVA (C) or two-way repeated measures ANOVA, followed by Bonferroni post-hoc test.

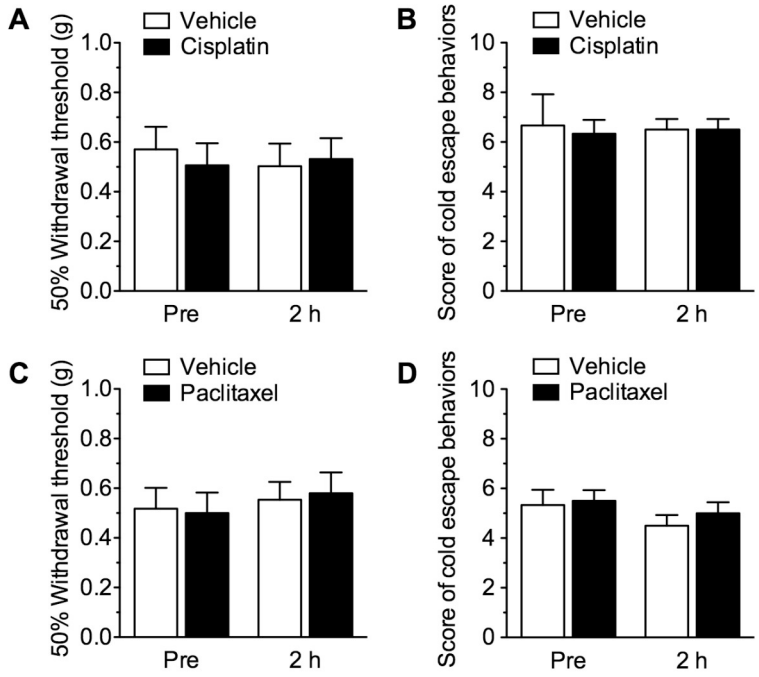

Figure 1-2. Neither cisplatin nor paclitaxel induces acute mechanical and cold hypersensitivity. Mice were intraperitoneally administered vehicle, cisplatin (5 mg/kg; **A**, **B**), or paclitaxel (6 mg/kg; **C**, **D**), and 2 h later the 50% withdrawal threshold to mechanical stimulation (**A**, **C**) and the escape behaviors in response to cold stimulation (**B**, **D**) were evaluated. Data are presented as the means ± S.E.M. of 6 mice.

Effects of analgesics on oxaliplatin-induced acute cold hypersensitivity

An NSAID, diclofenac (25 and 50 mg/kg) had no effect on the oxaliplatin-induced acute cold hypersensitivity (Fig. 1-3A). A strong opioid analgesic, morphine (5 and 10 mg/kg) tended to inhibit the cold hypersensitivity, although there was no significant difference when compared with vehicle-administered group (Fig. 1-3B). Tramadol (10 and 20 mg/kg) dose-dependently decreased the cold hypersensitivity. A significant difference was observed at 20 mg/kg when compared with vehicle-administered group (Fig. 1-3C). A calcium channel α_2-δ ligand, gabapentin (10 and 30 mg/kg) dose-dependently decreased the cold hypersensitivity. A significant difference was observed at 30 mg/kg when compared with vehicle-administered group (Fig. 1-3D). A tricyclic antidepressant, amitriptyline (5 and 10 mg/kg) had no effect on the cold hypersensitivity (Fig. 1-3E). An SNRI, milnacipran (10 and 30 mg/kg) tended to inhibit the cold hypersensitivity, although there was no significant difference when compared with vehicle-administered group (Fig. 1-3F). An orally available local anesthetic, mexiletine (10 and 30 mg/kg) dose-dependently decreased the cold hypersensitivity. A significant difference was observed at 30 mg/kg when compared with vehicle-administered group (Fig. 1-3G). Calcium gluconate (0.5 mmol/kg) infused before oxaliplatin administration significantly inhibited acute cold hypersensitivity, compared with vehicle-infused group (Fig. 1-3H).

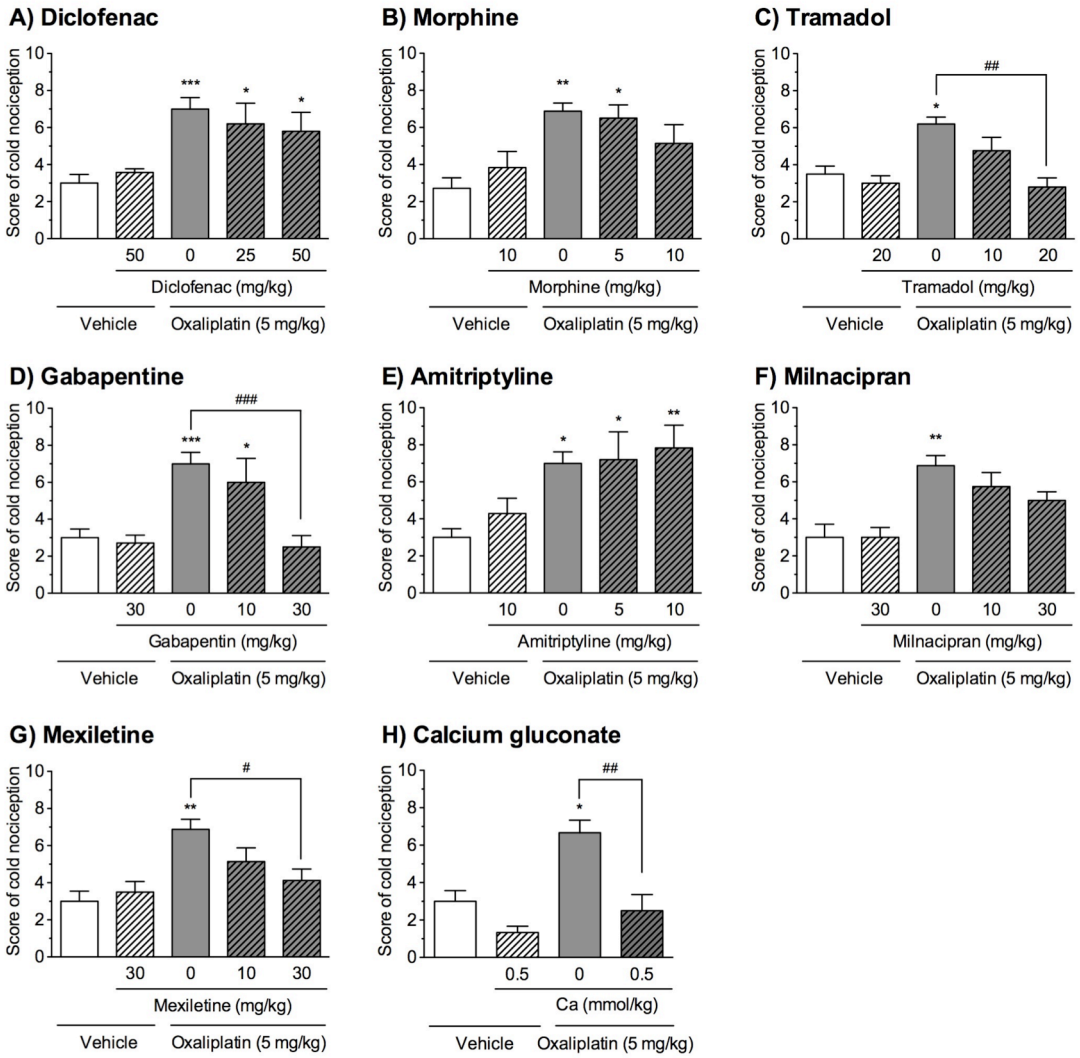

Figure 1-3. Effects of analgesics on oxaliplatin-induced acute cold hypersensitivity. Mice were given intraperitoneal administration of oxaliplatin (5 mg/kg). Cold plate tests were performed 2 hours after oxaliplatin administration. Morphine (5 and 10 mg/kg, *n* = 6-8), tramadol (10 and 20 mg/kg, *n* = 5-8), milnacipran (10 and 30 mg/kg, *n* = 4-8) and mexiletine (10 and 30 mg/kg, *n* = 5-8) were administered intraperitoneally 30 minutes before the tests. Diclofenac (25 and 50 mg/kg, *n* = 5-9), gabapentin (10 and 30 mg/kg, *n* = 4-9) and amitriptyline (5 and 10 mg/kg, *n* = 5-9) were administered intraperitoneally 60 minutes before the tests. Calcium gluconate (0.5 mmol/kg) was administered intravenously before oxaliplatin administration (*n* = 3-4). Data are presented as the means ± S.E.M. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to the group treated with vehicle alone instead of oxaliplatin. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (one-way ANOVA, followed by the Tukey-Kramer *post-hoc* test).

Discussion

The peripheral neuropathy caused by chemotherapeutic agents, including oxaliplatin, has been widely evaluated experimentally in rodents as hypersensitivity to mechanical and thermal stimuli in terms of mechanical allodynia and thermal hyperalgesia, respectively. Previous studies in animal models largely focus on the oxaliplatin-induced chronic painful neuropathy that appears several days to several weeks after oxaliplatin administration [11, 14, 23-25], while oxaliplatin-induced acute neuropathy is less well characterized [13]. My findings in mice, in which cold hypersensitivity was detected as early as 2 h after oxaliplatin administration, is consistent with the clinical observation of a characteristic acute sensory neuropathy triggered by cold that appears during or within hours of oxaliplatin infusion. By contrast, mechanical hypersensitivity in mice was observed 1 day, but not as early as 2 h, after drug administration and persisted for at least 7 days, consistent with previous reports [11, 13, 23]. Moreover, the rapid-onset cold hypersensitivity was not produced by another platinum-based chemotherapeutic agent, cisplatin, or by the non-platinum-containing chemotherapeutic agent, paclitaxel, both of which are known to induce chronic peripheral neuropathy following repeated administration [15, 20-22]. These findings suggest that the rapid-onset cold hypersensitivity is representative of the acute peripheral sensory neuropathy characteristic to oxaliplatin in mice and that the mouse model is suitable for exploring the mechanisms of this side effect and assessing effects of therapeutic drugs.

NSAIDs are representative broad-spectrum analgesics especially for nociceptive pain, while they are ineffective on neuropathic pain. Although NSAIDs are initially treated for the management of chemotherapy-induced painful peripheral neuropathy [8], it remains unclear whether acute peripheral neuropathy induced by oxaliplatin involves inflammatory components. The present results suggest that NSAIDs are ineffective on oxaliplatin-induced acute peripheral neuropathy.

It is reported that morphine (1-4 mg/kg) dose-dependently inhibits the chronic and subacute cold allodynia induced by oxaliplatin in rats [11, 12]. However, the present results suggest that morphine is less efficacious against oxaliplatin-induced acute peripheral neuropathy.

Tramadol exhibits analgesic effect through both μ-opioid receptor agonistic activity and antidepressant-like enhancement of descending serotoninergic and noradrenergic

pain inhibitory systems by blocking monoamine reuptake. The dual analgesic mechanisms are likely to contribute to its efficacy against neuropathic pain [26]. Indeed, it is clinically reported that tramadol/acetaminophen combination is effective in the management of oxaliplatin-induced painful neuropathy [27]. The present results further suggest that tramadol alleviates oxaliplatin-induced acute peripheral neuropathy probably due to its complementary and synergistic actions.

Gabapentin is often used as a first-choice drug for the management of neuropathic pain, as well as pregabalin [28]. However, gabapentin has not well proven efficacy in the treatment of oxaliplatin-induced peripheral neuropathy. Some clinical studies have not confirmed the ameliorating effect of gabapentin, while pregabalin reduced the severity of peripheral neuropathy in patients treated with oxaliplatin [9]. In animal models, gabapentin reduces oxaliplatin-induced chronic and/or subacute peripheral neuropathy [13, 29]. Furthermore, mRNA expression of Ca^{2+} channel $\alpha_2\delta$-1 subunit in the dorsal root ganglia is increased 10 days after oxaliplatin administration [29]. My present results suggest that gabapentin alleviates oxaliplatin-induced acute peripheral neuropathy.

Tricyclic antidepressants and SNRIs are widely used as first-choice drugs for the management of neuropathic pain [28]. Both antidepressants inhibit neuropathic pain by blocking noradrenaline and serotonin reuptake. However, their efficacy on the oxaliplatin-induced peripheral neuropathy is controversial [9]. In animal models, repeated administration of amitriptyline reduces oxaliplatin-induced chronic mechanical allodynia [30]. It is considered that disinhibition and imbalance of the descending serotonergic and noradrenergic pain inhibitory pathways contribute to neuropathic pain. However, oxaliplatin-induced acute peripheral neuropathy is unlikely to be mediated through the disinhibition and imbalance of these pathways, which may result in no and weak efficacy of these antidepressants. On the other hand, anti-allodynic effect of milnacipran is more potent than amitriptyline on cold allodynia in a neuropathic pain model rats [31], consistent with the present results.

Mexiletine blocks voltage-gated Na^+ channels, and is clinically used for painful diabetic neuropathy. An aspect of the oxaliplatin-induced peripheral neuropathy is mediated through voltage-gated Na^+ channels in sensory neurons [32]. In animal models, mexiletine dose-dependently inhibits the oxaliplatin-induced chronic cold allodynia [33]. Taken together, mexiletine is a potential treatment option for the oxaliplatin-induced

acute neuropathy.

Infusion of calcium and magnesium has been clinically treated for prevention and management of oxaliplatin-induced peripheral neuropathy, although a recent meta-analysis does not support them [10]. In animal models, pre-administration of calcium reduces the oxaliplatin-induced chronic and/or subacute cold hyperalgesia but not mechanical allodynia [13]. It is hypothesized that chelation of calcium by an oxaliplatin metabolite, oxalate, induces functional impairment of voltage-gated Na^+ channels, resulting in hyperexcitability of sensory neurons. Therefore, calcium infusion could enhance the closing rate of Na^+ channels, which may decrease the neuronal hyperexcitability induced by oxaliplatin [32]. The present results suggest that pre-infusion of calcium before oxaliplatin could prevent, at least, the oxaliplatin-induced acute peripheral neuropathy.

In conclusion, the present study suggests that the rapid-onset cold hypersensitivity is representative of the acute peripheral sensory neuropathy characteristic to oxaliplatin in mice. The data also provide evidences the efficacy of tramadol, Ca^{2+} channel α_2-δ ligands, Na^+ channel blockers and calcium gluconate, rather than opioid analgesics, antidepressants and NSAIDs, on the cold-triggered acute peripheral neuropathy induced by oxaliplatin.

Chapter 2 Involvement of thermal TRP channels in oxaliplatin-induced acute cold hypersensitivity

Oxaliplatin, widely used to treat advanced metastatic colorectal cancer, induces moderate to severe peripheral neuropathy, characterized by acute and chronic, two types of neurological symptoms [4, 5]. During or within hours after its infusion, an acute neuropathy, including acral numbness, paresthesia, dysesthesia and pain, develops in almost all patients that intensify over time, causing serious discomfort. While the oxaliplatin-induced chronic peripheral neuropathy can be explained, at least in part, by the accumulation of platinum adducts in the dorsal root ganglia (DRG) [34], the mechanisms underlying the acute peripheral neuropathy are poorly understood.

On the other hand, sensory neurons express several types of transient receptor potential (TRP) channels, including TRPV1, TRPA1, and TRPM8. These receptors are thermosensitive and play a critical role in pain generation [35, 36]. TRPV1 is activated by noxious heat, acidity, and noxious chemical stimuli such as capsaicin [37, 38]. TRPA1 is activated by noxious cold and a large number of irritants including allyl-isothiocyanate (AITC), cinnamaldehyde, allicin, and aldehydes, as well as oxygen, reactive-oxygen and nitrogen species [39-43]. TRPM8 is activated by innocuous as well as noxious cold, and by menthol, the ingredient of peppermint that produces its cooling sensation [44, 45]. Although still a matter of debate, recent evidence suggests that these thermosensitive TRP channels are responsible for chemotherapy-induced peripheral neuropathies. In rodents, oxaliplatin increases the expression of TRPA1 [14, 23, 24] and TRPM8 [25], but see [14, 24], but not TRPV1 [24], mRNAs in sensory ganglia. In mouse trigeminal ganglia, cisplatin increases TRPV1 and TRPA1 mRNAs levels [24], but see [46]. Oxaliplatin-induced mechanical and cold hypersensitivity is abolished by pharmacological inhibition or a gene-deficiency of TRPA1 [14, 23], TRPV1 [24], or TRPM8 [14, 25], but see [46], while cisplatin- and paclitaxel-induced painful neuropathy being inhibited by an antagonist or genetic deficiency of TRPV1 [20, 21, 24]. In those studies, chemotherapy-induced hypersensitivity was assessed several days to several weeks after a single or repeated administration of the compounds, which may reflect the subacute or chronic phase of chemotherapy-induced peripheral neuropathy.

In chapter 2, I investigated whether TRPA1, TRPM8, and TRPV1 are involved in the oxaliplatin-induced acute cold hypersensitivity *in vivo* and *in vitro*.

Methods

Animals

The male C57BL/6 J mice aged between 6-8 week-old were purchased from Japan SLC (Shizuoka, Japan). For experiments investigating the effects of TRPA1 deficiency, wild-type (*Trpa1*$^{+/+}$) and homozygous (*Trpa1*$^{-/-}$) mouse littermates from heterozygous/heterozygous *Trpa1*$^{+/-}$ mice were used (6- to 8-weeks-old). *Trpa1*$^{-/-}$ mice bred from heterozygous mice with a C57BL/6 × 129 S1 background were obtained from Jackson Laboratory (Bar Harbor, ME) and genotyped as previously described [47]. The *Trpa1*$^{-/-}$ mouse line was backcrossed to C57BL/6 J mice for at least 10 generations. All mice were housed under constant ambient temperature (24 ± 1°C) and humidity (55 ± 10%), with alternate light-dark cycles from 8:00 a.m. to 20:00 p.m.. Food and water were freely available. All experiments were conducted in accordance with the Ethical Guidelines of the Kyoto University Animal Experimentation Committee.

Drugs

Oxaliplatin and sodium oxalate were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and freshly dissolved in 5% glucose solution. *cis*-Diammineplatinum(II) dichloride (cisplatin) and paclitaxel were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Cisplatin was freshly dissolved in sterile saline, and paclitaxel in Cremophor® EL (Sigma-Aldrich) and dehydrated ethanol (1:1) to obtain a stock solution. Prior to its administration, paclitaxel was further diluted with sterile saline. Mice received a single intraperitoneal administration of oxaliplatin (1, 5 or 10 mg/kg), cisplatin (5 mg/kg), paclitaxel (6 mg/kg), or vehicle. The doses of these chemotherapeutic agents were chosen based on previous reports with a single intraperitoneal administration [11, 13-17]. HC-030031 (100 mg/kg, Enzo Life Sciences, Exeter, UK) was prepared in 0.5% methylcellulose (Wako).

Behavioral tests

Cold plate test

Cold sensitivity was assessed with the hot/cold plate (Ugo Basile, Milan, Italy). Mice were allowed to acclimate to the testing apparatus for 1 h, after which they were individually placed on the center of a cold plate maintained at 5°C in a transparent Plexiglas cylinder. Escape behaviors were observed for 60 s and graded with a score of 0 = no response; 1 = moderate effort to avoid cold, such as lifting a hindpaw or walking backwards; and 2 = vigorous effort to escape cold, such as jumping. The sum of the scores recorded within a 60-s period was calculated.

TRP channel agonist-evoked nocifensive behaviors

DL-Menthol (Sigma-Aldrich) and capsaicin (Nacalai Tesque, Kyoto, Japan) were dissolved in dimethyl sulfoxide (DMSO) as a stock solution (800 mg/ml and 80 mg/ml, respectively). Allyl-isothiocyanate (AITC, Wako), menthol, and capsaicin were diluted in corn oil (Sigma-Aldrich). The mice were allowed to acclimate in a clear acrylic cylinder for at least 40 min after which 20 μl of AITC (0.1%), capsaicin (1.6 μg) or menthol (160 μg) was subcutaneously injected into the plantar surface of the left hindpaw. AITC- and capsaicin-evoked nocifensive behaviors were measured as the durations of consecutive licking and flicking behaviors for 20 min and 5 min, respectively. Menthol-evoked nocifensive-like behaviors (i.e., hindpaw lifting and backwards walking) were scored as for the cold plate test described above. The sum of the scores recorded within a 5-min observation period was calculated.

Primary cultures of DRG neurons

Bilateral L1-L6 DRGs were harvested from two freshly killed adult male C57BL/6 J mice. DRGs were incubated for 1 h at 37°C in Hank's balanced salt solution (137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.6 mM D-glucose, 2.4 mM HEPES, 25 mM glucose, pH 7.4) containing 0.3% collagenase and 0.4% dispase. A Percoll (Sigma-Aldrich) gradient was used to separate myelin and nerve debris from DRG neurons as follows: Solutions of 30% and 60% Percoll were prepared with L15 medium. The 30% Percoll was gently layered over the 60% Percoll solution,

and the cell suspension over the Percoll gradient. After 10 min of centrifugation at 1800 × g, the cells were harvested from the Percoll interface and suspended in 8 ml L15 medium, then centrifuged again for 5 min at 1800 × g. The supernatant was removed and the cell pellet resuspended in 70 µl Dulbecco's modified Eagle medium (DMEM), containing 10% heat-inactivated fetal bovine serum, penicillin G (100 U/ml), and streptomycin (100 µg/ml), followed by plating onto laminin-coated coverslips (3 mm × 7 mm) and incubation at 37°C. After 4 h incubation, 1.5 ml DMEM was added and the cells were incubated again, this time overnight at 37°C.

Fluorometric Ca^{2+} imaging

Cultured DRG neurons on coverslips were loaded for 30 min with 5 µM fura-2/AM (Dojindo, Kumamoto, Japan) in Krebs-Ringer solution (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, 10 mM HEPES) containing 0.01% cremophore EL (Sigma-Aldrich). The cells were then washed with Krebs-Ringer solution and transferred to an imaging chamber. The AQUACOSMOS/ORCA-AG imaging system (Hamamatsu Photonics, Shizuoka, Japan) was used to capture the fluorescence images obtained with alternating excitation at 340 and 380 nm and emission at > 510 nm. Emission ratios (F_{340}/F_{380}) were calculated for each 5-s interval after subtraction of the background. The cells showing the F_{340}/F_{380} ratio of over 1.4 at the baseline were excluded. A cellular response to a drug was defined as an increase in the F_{340}/F_{380} ratio by more than 0.2 during the 3-min application period. All experiments were performed at room temperature. Oxaliplatin stock solution (5 mM) was prepared in sterile water and further diluted in DMEM to 100 µM before every experiment. AITC (100 mM), menthol (1 M), and capsaicin (100 mM) were prepared as stock solutions in DMSO and diluted in Krebs-Ringer solution.

Statistical analysis

The data were analyzed using Graphpad Prism and are presented as means ± S.E.M. Statistical significance was calculated by one-way or two-way analyses of variance (ANOVA), followed by the Bonferroni post-hoc test. In all cases, differences of $p < 0.05$ were considered statistically significant.

Results

Effects of oxaliplatin, cisplatin, and paclitaxel on AITC-, menthol- and capsaicin-evoked nocifensive behaviors

To investigate the involvement of thermosensitive TRP channels, I assessed the effects of oxaliplatin, cisplatin, and paclitaxel on the nocifensive behaviors evoked by TRP channel agonists. Intraplantar (i.pl.) injection of AITC (0.1%, 20 μl), a TRPA1 agonist, evoked nocifensive behaviors such as licking and flicking of the injected hindpaw, whereas this was not the case following i.pl. vehicle injection. When mice were preinjected with oxaliplatin (1, 5 and 10 mg/kg, i.p.) and then tested 2 h later, the duration of the AITC-evoked nocifensive behaviors were significantly enhanced in a dose-dependent manner ($F_{3,31} = 5.711$, $p < 0.001$). The significant differences were observed at doses of 5 and 10 mg/kg, but not 1 mg/kg, compared with mice preinjected with vehicle (Fig. 2-1A). I examined the time course of the oxaliplatin-induced enhancement of the AITC-evoked nocifensive behaviors. The duration of the AITC-evoked nocifensive behaviors were significantly enhanced even 1 and 3 days, but not 7 days after a single administration of oxaliplatin (5 mg/kg, i.p.), compared with mice preinjected with vehicle (Fig. 2-1B). Intraplantar injection of menthol (160 μg), a TRPM8/TRPA1 agonist [48], evoked nocifensive-like behaviors, such as backwards walking and lifting of the injected hindpaw. In mice preinjected with oxaliplatin 2 h prior to testing, the scores of menthol-evoked nocifensive-like behaviors were significantly higher than those of vehicle-administered mice (Fig. 2-1C). The enhancement of menthol-evoked nocifensive-like behaviors induced by oxaliplatin pretreatment was significantly inhibited in *Trpa1*$^{-/-}$ mice to the scores in vehicle-pretreated mice (Fig. 2-1D). Intraplantar injection of the TRPV1 agonist capsaicin (1.6 μg) evoked nocifensive behaviors such as licking and flicking of the injected hindpaw. There was no significant difference in the duration of the capsaicin-evoked nocifensive behaviors between vehicle- and oxaliplatin-preinjected mice (Fig. 2-1E).

When mice were preinjected with oxalate (1.7 mg/kg, i.p.) and then tested 2 h later, the duration of the AITC-evoked nocifensive behaviors was significantly enhanced compared with mice preinjected with vehicle (Fig. 2-1F).

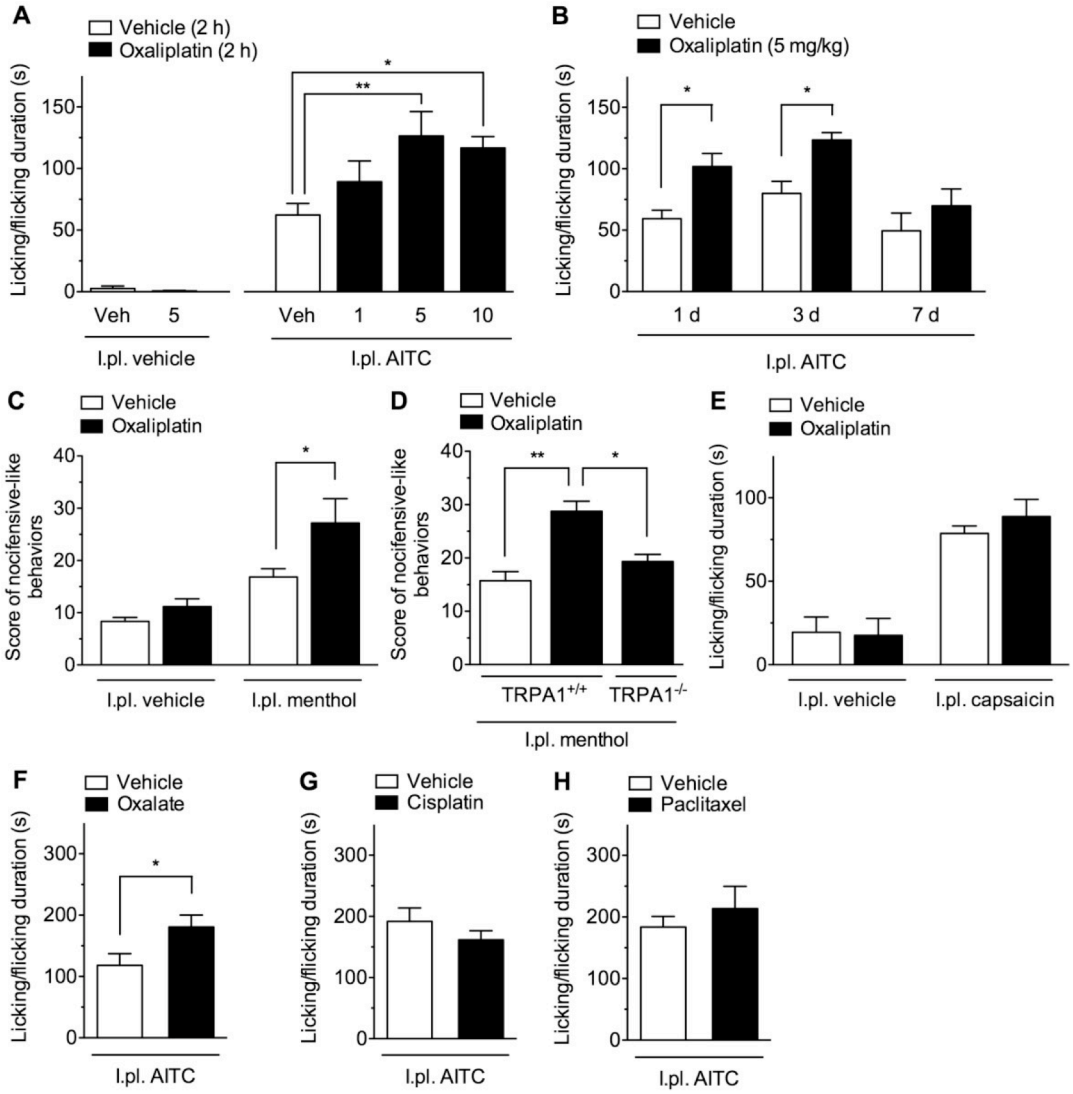

Figure 2-1. A single administration of oxaliplatin, but not cisplatin and paclitaxel, enhances AITC-evoked nocifensive behaviors. (**A**) Two hours after a single administration of vehicle or oxaliplatin (1, 5 or 10 mg/kg), mice received an intraplantar injection of vehicle (*n* = 3) or AITC (0.1%, 20 µl; *n* = 7-14). (**B**) 1, 3 or 7 days after a single administration of vehicle or oxaliplatin (5 mg/kg), mice received an intraplantar injection of AITC (0.1%, 20 µl). *n* = 5-6. (**C**) Two hours after a single administration of vehicle or oxaliplatin (5 mg/kg), mice received an intraplantar injection of vehicle or menthol (1.6 µg, 20 µl). *n* = 6. (**D**) $TRPA1^{+/+}$ or $TRPA1^{-/-}$ mice were intraperitoneally administered vehicle or oxaliplatin (5 mg/kg) 2 h before the menthol-evoked nocifensive behaviors. *n* = 3-4. (**E**) Two hours after a single administration of vehicle or oxaliplatin (5 mg/kg), mice received an intraplantar injection of vehicle or capsaicin (160 µg, 20 µl). *n* = 6. (**F**-**G**) Two hours after a single administration of oxalate (1.7 mg/kg; **F**), cisplatin (5 mg/kg; **G**), paclitaxel (6 mg/kg; **H**) or vehicle, mice received an intraplantar injection of AITC (0.1%, 20 µl). *n* = 6-7. AITC- and capsaicin-evoked nocifensive behaviors were measured for 20 min and 5 min, respectively; menthol-evoked nocifensive-like behaviors were scored for 5 min. Data are presented as the means ± S.E.M. * $p < 0.05$; ** $p < 0.01$.

By contrast, a 2-h pre-injection of cisplatin (5 mg/kg, i.p.) or paclitaxel (6 mg/kg, i.p.) had no effects on the duration of the AITC-evoked nocifensive behaviors (Fig. 2-1G, H).

Oxaliplatin enhances the response to TRPA1 agonist, but not that of TRPM8 and TRPV1 agonists, in cultured DRG neurons

The effects of oxaliplatin pretreatment on the responses to TRPA1, TRPM8, and TRPV1 agonists in cultured DRG neurons isolated from naïve mice were assessed by calcium imaging. An application of the TRPA1 agonist AITC at concentrations of 1, 10 and 100 μM to naïve cultured DRG neurons concentration-dependently evoked a $[Ca^{2+}]_i$ increase in approximately 5.0 ± 1.3%, 22.2 ± 3.2% and 38.2 ± 2.8% of the cells, respectively, consistent with previous reports (25-45%) [48-50]. To better detect alterations of TRPA1 function, cultured DRGs were treated with a relatively low AITC concentration of 10 μM. In cultured DRG neurons pretreated with oxaliplatin (30, 100 and 300 μM) for 2 h, the numbers of 10 μM AITC-sensitive cells were increased in a concentration-dependent manner, although the amplitudes of increase in F_{340}/F_{380} ratio seemed to be less or not increased (Fig. 2-2). I quantitatively assessed the numbers of AITC-sensitive cells. In cultured DRG neurons pretreated with oxaliplatin (30 μM) for 1, 2 and 4 h, the numbers of AITC-sensitive cells were not changed, compared with those pretreated with vehicle for 1, 2 and 4 h ($F_{1,30}$ = 2.55, P = 0.121) (Fig. 2-3A). By contrast, the numbers of AITC-sensitive cells pretreated with oxaliplatin (100 or 300 μM) for 1, 2 and 4 h were significantly increased ($F_{1,24}$ = 14,24, $P < 0.001$ and $F_{1,30}$ = 18.85, $P < 0.001$, respectively). Although the changes became apparent at 1 h, significant differences reached at 2 and 4 h of oxaliplatin pretreatment (Fig. 2-3B, C).

An application of menthol (100 μM) evoked a $[Ca^{2+}]_i$ increase in 4-6% of cultured DRG neurons pretreated with vehicle, which was consistent with (4.2-7% [51-53]) or less than previous reports (10-17% [44, 46, 49, 54, 55]). In cultured DRG neurons pretreated with oxaliplatin (100 μM) for 1, 2, or 4 h, there was no change in the number of menthol-sensitive cells at any time point (Fig. 2-3D). Similarly, an application of capsaicin at a relatively low concentration of 500 nM evoked a $[Ca^{2+}]_i$ increase in 22-29% of vehicle-treated cultured DRG neurons, with no change in the number of capsaicin-sensitive cells at any time point in those pretreated with oxaliplatin (Fig. 2-3E).

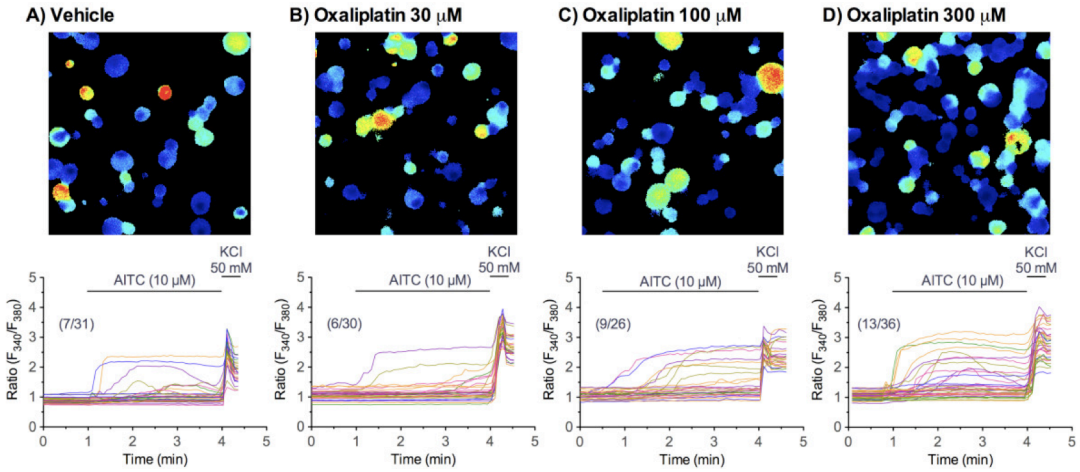

Figure 2-2. Oxaliplatin pretreatment increases the AITC-evoked Ca^{2+}response in cultured DRG neurons. The cultured neurons were pretreated with vehicle (**A**) or oxaliplatin at concentrations of 30 (**B**), 100 (**C**) or 300 μM (**D**) for 2 h after which AITC (10 μM) was added for 3 min. Representative image of fluorescence (upper panels) and Ca^{2+} concentration (F_{340}/F_{380} ratio) recorded in individual cultured DRG neurons (lower panels). All neurons in the fields were identified based on the $[Ca^{2+}]_i$ increase elicited by the application of 50 mM KCl. Cell numbers in (AITC-sensitive DRG neurons)/(Total counted neurons) in the representative data are indicated in parentheses in lower panels.

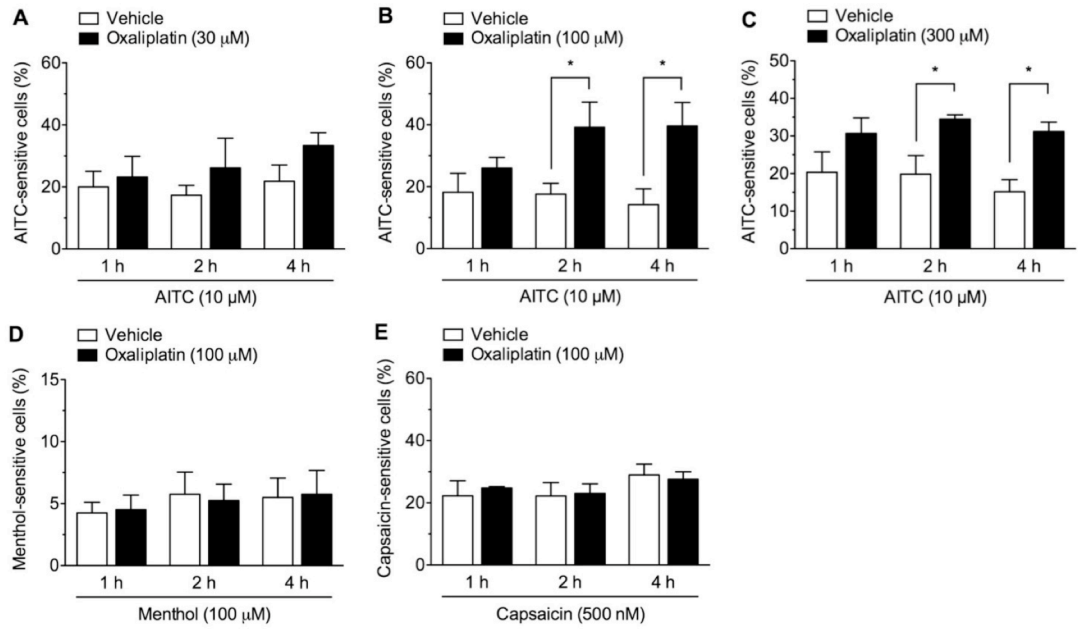

Figure 2-3. Oxaliplatin pretreatment increases the number of AITC-sensitive DRG neurons. (**A**-**C**) In cultured DRG neurons pretreated with vehicle or oxaliplatin at concentrations of 30 (**A**), 100 (**B**) or 300 μM (**C**) for 1, 2, or 4 h, AITC (10 μM) was added for 3 min. (**D**, **E**) In cultured DRG neurons pretreated with vehicle or oxaliplatin (100 μM) for 1, 2, or 4 h, menthol (100 μM; **D**) or capsaicin (500 nM; **E**) was added for 3 min, and the Ca^{2+} responses of the neurons were determined. Cells were considered responsive if their F_{340}/F_{380} ratio increased by more than 0.2 during the 3-min application. The values show the percentage of agonist-sensitive cells in 50 mM KCl-positive neurons. Data are presented as the mean ± S.E.M. of 4-6 separate experiments. *p < 0.05, compared with corresponding vehicle-pretreatment.

Involvement of TRPA1 in acute oxaliplatin-induced cold hypersensitivity

To determine whether TRPA1 is involved in acute oxaliplatin-induced cold hypersensitivity, I examined the effects of a TRPA1 antagonist and of TRPA1 deficiency. In the cold plate test, escape behavior scores measured in response to cold stimulation were significantly higher in mice tested 2 h after oxaliplatin (5 mg/kg, i.p.) administration than in mice tested 2 h after vehicle administration. In 2-h vehicle-administered mice, an injection of the TRPA1 antagonist HC-030031 (100 mg/kg, i.p.) 30 min before the cold plate test tended to decrease the cold escape behavior scores, although the effect was not significant. In 2-h oxaliplatin-administered mice, HC-030031 significantly inhibited oxaliplatin-induced acute cold hypersensitivity compared to 30-min vehicle-injected mice (Fig. 2-4A). Similarly, *Trpa1*$^{+/+}$ mice exhibited acute cold hypersensitivity 2 h after oxaliplatin (5 mg/kg) administration, whereas the response was completely abolished in *Trpa1*$^{-/-}$ mice (Fig. 2-4B).

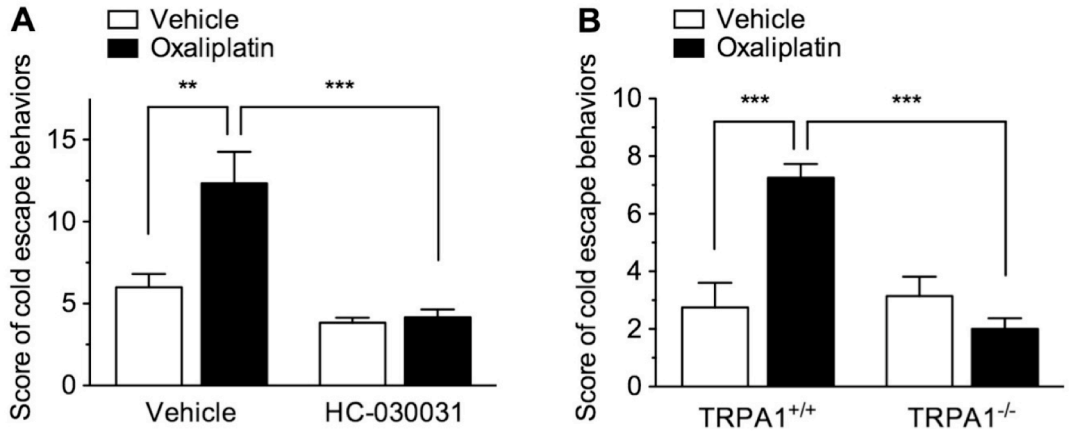

Figure 2-4. TRPA1 is involved in oxaliplatin-induced acute cold hypersensitivity. (**A**) Mice were intraperitoneally administered vehicle or oxaliplatin (5 mg/kg) 2 h before the cold plate test, with vehicle or HC-030031 (100 mg/kg) intraperitoneally injected 30 min before testing. n = 6. (**B**) TRPA1$^{+/+}$ or TRPA1$^{-/-}$ mice were intraperitoneally administered vehicle or oxaliplatin (5 mg/kg) 2 h before the cold plate test. n = 7-8. Escape behaviors in response to cold stimulation (5°C) were scored in a cold plate test. Data are presented as the mean ± S.E.M. ** p < 0.01, *** p < 0.001.

Discussion

In the present study, I provide the first evidence that TRPA1 in DRG neurons mediates the acute phase of oxaliplatin-induced peripheral neuropathy, as supported by the following results. 1) A single administration of oxaliplatin, as well as its metabolite oxalate, produced rapid-onset cold hypersensitivity within 2 h; this response was blocked by a TRPA1 antagonist and by TRPA1 deficiency. 2) Nocifensive behaviors evoked by AITC and menthol, but not by capsaicin, were enhanced 2 h after oxaliplatin administration. 3) Pretreatment of the cultured DRG neurons with oxaliplatin for 2-4 h increased the number of AITC-, but not of menthol- and capsaicin-sensitive neurons.

The major finding of this study is that oxaliplatin leads to the selective enhancement of TRPA1-mediated responses within a relatively short time, both in vivo and in vitro. AITC-evoked nocifensive behaviors and Ca^{2+} influx in DRG neurons are mediated through the activation of TRPA1 [47, 48]. Oxaliplatin increased both of these TRPA1-mediated responses within several hours, suggesting that it rapidly leads to an enhanced TRPA1 responsiveness in sensory neurons. The finding that oxalate enhanced AITC-evoked nocifensive behaviors suggests that the rapid-onset effects characteristic of oxaliplatin are caused by its metabolite, oxalate, or by an oxalate-related structure of oxaliplatin. Consistent with my findings, Sakurai et al. showed that both oxaliplatin and oxalate induce an early-phase (several hours) cold hyperalgesia in the acetone test, whereas a late-phase mechanical allodynia is induced by oxaliplatin or its another metabolite dichloro(1,2-diaminocyclohexane)platinum, but not oxalate, in rats [13]. On the other hand, the oxaliplatin-induced enhancement of AITC-evoked nocifensive behaviors lasted, at least, 3 days after the administration, suggesting that the enhanced TRPA1 responsiveness contributes to not only the acute (several hours), but also, at least, subacute (several days) oxaliplatin-induced cold hypersensitivity.

Despite some controversies, recent evidence points to the involvement of TRPA1 in oxaliplatin-induced subacute and chronic peripheral neuropathy [14, 23, 24, 56]. As previous studies shown, subacute (several days) mechanical and cold hypersensitivities-induced by a single administration of oxaliplatin were inhibited by either a TRPA1 antagonist or TRPA1 deficiency [14, 23], while the antagonist failed to inhibit the oxaliplatin-enhanced cold-temperature avoidance behavior [14]. Furthermore, subacute (several days) and chronic (several weeks) administration of oxaliplatin

increases TRPA1 mRNA levels in DRGs and in the trigeminal ganglion [14, 24]. Controversially, a transient up-regulation of TRPA1 mRNA is observed in DRGs only 6 h after a single administration of oxaliplatin [23]. However, it is unlikely that oxaliplatin is able to increase the expression of functional TRPA1 protein within several hours of its administration. In the Ca^{2+} imaging experiments of the present study, the AITC concentration was set relatively low (10 μM). Nonetheless, following oxaliplatin treatment, 10 μM AITC produced nearly the same proportion of sensitive neurons (approximately 40%) as obtained with the submaximal concentration of AITC (100 μM) used in the control. Therefore, it is likely that oxaliplatin acutely produces an enhanced TRPA1 responsiveness by increasing the sensitivity to AITC, i.e., through the sensitization of existing TRPA1 and not by an increase in the number of TRPA1-expressing cells. Under inflammatory conditions, TRPA1 sensitization involves its translocation to the plasma membrane via phospholipase C and protein kinase A (PKA) signaling [57, 58]. Acute oxaliplatin may likewise induce the TRPA1 sensitization via PKA signaling [56] or through other mechanisms specific to oxaliplatin and oxalate.

TRPM8 is expressed in a subpopulation of small-diameter sensory neurons, which correlates with responses to cooling and menthol [45, 59, 60]. Menthol sensation is mainly ascribed to TRPM8, while it also activates TRPA1 in a bimodal manner [48]. In Ca^{2+} imaging experiments, menthol-sensitive DRG neurons are largely abolished in $TRPM8^{-/-}$ mice, although a small population of menthol-sensitive neurons remains, probably via TRPA1 activation [51, 61]. Nevertheless, I found no change in the number of menthol-sensitive DRG neurons in response to oxaliplatin, suggesting that it has no acute effect on TRPM8-mediated responses. Although menthol-sensitive DRG neurons mediated thorough TRPA1 activation might be increased by acute oxaliplatin, they may be undetectable probably due to too small population or weak activation of TRPA1 by 100 μM menthol in a bimodal phase [48]. Supporting my findings, oxaliplatin enhances Ca^{2+} responses to icilin (TRPA1/TRPM8 agonist), but not WS12 (TRPM8 selective agonist), in rat DRG neurons [56]. By contrast, my present findings showed that menthol-evoked nocifensive-like behaviors were enhanced after acute oxaliplatin administration, which was inhibited by TRPA1 deficiency. Since it is possible that menthol-evoked nocifensive-like behaviors are mediated through TRPA1 activation [62], they may be increased by the enhanced responsiveness of TRPA1 after acute

oxaliplatin administration in the dose of menthol used in this study. Several studies examine the involvement of TRPM8 in oxaliplatin-induced peripheral neuropathy. The subacute, but not chronic, effects of oxaliplatin administration were shown to include a transient up-regulation of TRPM8 mRNA in DRGs [14, 24, 25]. In TRPM8$^{-/-}$ mice, oxaliplatin-enhanced cold avoidance is abolished, while there is no change in oxaliplatin-induced mechanical hypersensitivity [14]. Pharmacological blockade of TRPM8 has no effect on oxaliplatin-induced subacute cold hypersensitivity [46]. Thus, although TRPM8 involvement in oxaliplatin-induced subacute peripheral neuropathy remains to be clarified, my findings seem to rule out an important role for TRPM8 in oxaliplatin-induced acute peripheral neuropathy

A body of evidence suggests that TRPV1 plays a role in chemotherapy-induced chronic peripheral neuropathy [20, 21, 34], similar to the neuropathic pain induced by peripheral nerve injury [63]. However, in the present study, neither cisplatin nor paclitaxel altered capsaicin-evoked, TRPV1-mediated nocifensive behaviors. Furthermore, oxaliplatin had no rapid-onset effect on capsaicin-evoked nocifensive behaviors or the number of capsaicin-sensitive DRG neurons, suggesting that oxaliplatin-induced acute peripheral neuropathy is not mediated by TRPV1. Consistent with the present findings, other studies did not find evidence of TRPV1 involvement in oxaliplatin-induced subacute and chronic cold hypersensitivity [23-25].

Accumulating evidence suggests that oxaliplatin, as a platinum-based drug like cisplatin, induces chronic peripheral neuropathy by its direct and indirect neurotoxic effects on peripheral sensory neurons [5, 23]. The painful neurotoxicity may secondarily up-regulate and/or sensitize TRPA1, TRPV1, and TRPM8, as occurs in nerve injury-induced neuropathic pain [63-65]. However, our results indicate that oxaliplatin leads to a rapid, preferentially enhanced responsiveness of TRPA1. Since the rapid effect of the drug is unlikely to be due to its neurotoxicity on sensory neurons, a more likely explanation is an alteration in TRPA1 function, either directly or indirectly, within several hours, although the mechanisms remain unclear.

Taken together, these results suggest that a brief treatment with oxaliplatin or its metabolite oxalate is sufficient to enhance the responsiveness of TRPA1 but not that of TRPM8 and TRPV1 expressed by DRG neurons, which may contribute to the characteristic acute peripheral neuropathy induced by oxaliplatin.

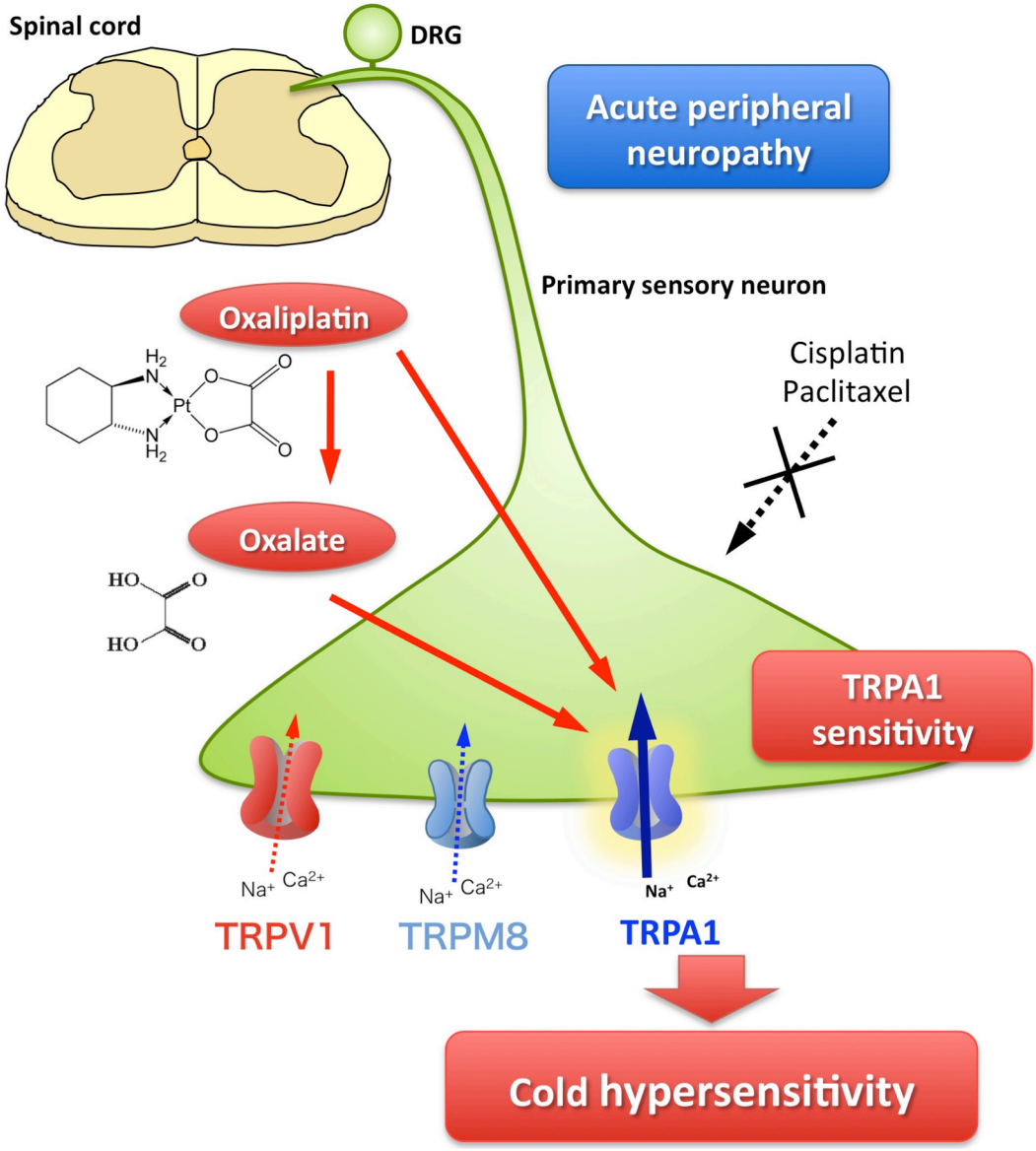

Figure 2-5. Schematic diagram depicting the involvements of thermal TRP channel in oxaliplatin-induced acute cold hypersensitivity

A brief treatment with oxaliplatin or its metabolite oxalate is sufficient to enhance the responsiveness of TRPA1 but not that of TRPM8 and TRPV1 expressed by DRG neurons, which may contribute to the characteristic acute peripheral neuropathy induced by oxaliplatin.

Chapter 3 Mechanisms of TRPA1 activation in oxaliplatin-induced acute cold hypersensitivity

Oxaliplatin, a platinum-based chemotherapeutic agent, causes peculiar acute peripheral neuropathy. Oxaliplatin-induced acute peripheral neuropathy appears in almost all patients rapidly after infusion and is triggered or exacerbated by cold, but the mechanisms are poorly understood. I hypothesized that TRPA1, a cation channel activated by oxidative stress and cold temperature, contributes to acute cold hypersensitivity induced by oxaliplatin. Recently, I have established a mouse model of rapid-onset cold hypersensitivity induced by oxaliplatin and reported that acute cold hypersensitivity induced by oxaliplatin is caused by the enhanced responsiveness of TRPA1 in mice. In this study, I further explored the mechanisms how TRPA1 is activated and/or sensitized by oxaliplatin.

In Ca^{2+} imaging and patch-clamp using HEK293 cells expressing human TRPA1, high concentrations of oxaliplatin (100-1000 μM) evoked Ca^{2+} response and increased whole-cell currents, respectively, but not in mock-transfected cells. The oxaliplatin-induced Ca^{2+} response was abolished by a TRPA1 antagonist, HC-030031, an antioxidant, glutathione and a reactive oxygen species (ROS) scavenger, PBN. To determine the site of action to oxaliplatin on TRPA1, I used several mutated TRPA1 mutating cysteine residues to serine and examined the responsiveness of these mutant clones in oxaliplatin-induced TRPA1 activation by Ca^{2+} imaging test. I found that oxaliplatin activated TRPA1 through oxidative cysteine modification, which perhaps mediated by hydrogen peroxide (H_2O_2). Moreover, in HEK293 cells preloaded with H_2O_2-specific indicator PG1, I confirmed that oxaliplatin increased H_2O_2 production. These data suggest that high concentration of oxaliplatin-induced TRPA1 activation is mediated through ROS production.

However, the concentration of oxaliplatin used in whole cell pacth clamp method and Ca^{2+} imaging test was 1 mM, which is higher than blood concentration of oxaliplatin. Thus, I supposed there might be other mechanisms of oxaliplatin to sensitize but not activate TRPA1. Next, I investigated the effects of a relatively lower concentration of oxaliplatin pretreatment on H_2O_2-evoked TRPA1 responses. In TRPA1-expressed HEK293 cells, pretreatment with 100 μM oxaliplatin for 2 h increased H_2O_2 (10 μM)-evoked Ca^{2+} response. In cell-attached patches, number of

open channels x open probability (NPo) of TRPA1 channel was significantly increased after oxaliplatin pretreatment. Finally, when mice were treated with oxaliplatin (5 mg/kg) for 2 h, nocifensive behaviors evoked by intraplantar injection of H_2O_2 (0.5%, 20 μl) were significantly enhanced.

What is the mechanism of oxaliplatin-induced TRPA1 sensitization? Although some studies show TRPA1 mRNA is increased in DRGs after oxaliplatin administration, it is unlikely that oxaliplatin is able to increase the expression of functional TRPA1 protein within several hours of its administration. Interestingly, some group demonstrates TRPA1 membrane levels are increased by AITC stimuli, suggesting TRPA1 translocation to the membrane might represent one of the mechanisms controlling TRPA1 functionality upon acute activation. Therefore, I also investigated the whether TRPA1 membrane levels would change after oxalipaltin treatment in TRPA1-transfected HEK293 cells (data not shown). However, TRPA1 membrane levels were not changed after oxaliplatin pretreated for several hours, compared to vehicle-pretreated group. Thus, I considered that the increase of total TRPA1 protein expression or TRPA1 membrane levels have little effect on oxaliplatin-induced TRPA1 sensitization.

The present study reveals mechanisms of TRPA1 in oxaliplatin-induced acute cold hypersensitivity, as supported by the following results. 1) TRPA1 is activated by high concentration oxaliplatin; this response was inhibited by cysteine residues mutations and also by antioxidant or ROS scavenger. 2) On the other hand, low concentration oxaliplatin sensitize TRPA1 to ROS; which was proved by pretreatment of low concentration oxalipaltin enhanced the H_2O_2-evoked TRPA1 responses *in vivo* and *in vitro*.

Taken together, these results suggest oxaliplatin could sensitize TRPA1 function, and subsequently the sensitized TRPA1 is activated by ROS probably produced from platinum-based chemotherapeutic agent-mediated mitochondrial dysfunction, which may contribute to the oxaliplatin-induced acute peripheral neuropathy.

Summary

In this study, I explored the mechanisms of oxaliplatin-induced acute peripheral neuropathy as follows:

In chapter 1, I established a new oxaliplatin-induced acute cold hypersensitivity mouse model. A single intraperitoneal administration of oxaliplatin induced a characteristic acute cold hypersensitivity, while mechanical hypersensitivity was not observed, which is similar with the clinical observation. Then the effects of standard analgesics on the oxaliplatin-induced cold hypersensitivity were evaluated in this mouse model. I found that gabapentin, mexiletine, tramadol and calcium gluconate significantly inhibited, and morphine and milnacipran decreased the acute cold hypersensitivity, while diclofenac and amitriptyline had no effects. These results suggest that gabapentin, mexiletine and calcium gluconate are effective against oxaliplatin-induced acute peripheral neuropathy.

In chapter 2, the involvements of thermosensitive TRP channels were evaluated. Pre-treatment of oxalipaltin enhanced TRPA1 channel agonist-evoked nocifensive behaviors and $[Ca^{2+}]_i$ responses in cultured DRG neurons, respectively. Moreover, the oxaliplatin-induced cold hypersensitivity was inhibited by TRPA1 knockout mice and a TRPA1 antagonist, HC030031. The present data suggest that the acute cold hypersensitivity characteristically induced by oxaliplatin could be linked to an enhanced responsiveness of TRPA1, but not TRPM8 and TRPV1, on DRG neurons.

In chapter 3, the mechanisms of TRPA1 activation/sensitization were further investigated. High concentration of oxaliplatin elicited TRPA1 activation directly, while low concentration of oxaliplatin pretreatment enhanced hydrogen peroxide-evoked responses. Taken together, these results suggest oxaliplatin could sensitize TRPA1 function, and subsequently the sensitized TRPA1 is activated by ROS probably produced from oxaliplatin-mediated mitochondrial dysfunction, which may contribute to the oxaliplatin-induced acute peripheral neuropathy.

In conclusion, present study highlights TRPA1 plays a critical role in oxaliplatin-induced acute cold hypersensitivity and TRPA1 antagonists as a promising new approach for the treatment of oxaliplatin-induced acute peripheral neuropathy.

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Publications

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Acute cold hypersensitivity characteristically induced by oxaliplatin is caused by the enhanced responsiveness of TRPA1 in mice.

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Meng Zhao, Saki Nakamura, Takahito Miyake, Kanako So, Hisashi Shirakawa, Shogo Tokuyama, Minoru Narita, Takayuki Nakagawa, Shuji Kaneko

Pharmacological characterization of standard analgesics on oxaliplatin-induced acute cold hypersensitivity in mice

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Mechanism of TRPA1 activation in oxaliplatin-induced acute peripheral neuropathy

Prepare for submission

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